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(54) ## Method of making uniformly sized liposomes and liposomes so made.

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Description

This invention relates to liposomes. These are used as a vehicle for administration of drugs. It is particularly (though not exclusively) concerned with the incorporation into such liposomes of bis-anthracyclines, which have utility in inhibiting DNA function and therefore as anti-cancer drugs. Such compounds are disclosed and claimed in European Application 79300470.6 (now European Patent No. 4467) from which the present application is divided.

Liposomes are lipid micro-vesicles of approximately spherical shape. The outer shell of a liposome consists of a phospholipid bilayer which encloses a volume of water, an aqueous solution or partly aqueous solution. Liposomes with only one lipid shell are designated unilamellar vesicles; those with additional lipid shells, like layers of an onion, are called multi-lamellar vesicles. Either type may be small, e.g., 150—400 nm in diameter, or large, e.g., up to the size of red blood cells. A large liposome may contain many times the volume of a small liposome.

Liposomes may be produced by hydration and mechanical dispersion of dried lipoidal material in an aqueous solution. The lipoidal material can be phospholipids or other lipids, cholesterol and its derivatives or a variety of amphiphiles including macromolecules or mixtures of these. However, liposomes prepared this way are mixtures of all the types noted above, with a variety of dimensions, compositions and behaviours. This unpredictable variety leads to inconsistent measures of liposome properties and unreliable characterizations. To reduce the heterogeneity of mechanically dispersed liposomes, such dispersions may be filtered through a membrane filter (see FR 2298318A) exposed to sonication which decreases average liposome size. Under extensive sonication, occasionally populations of liposomes are reduced to small unilamellar vesicles, but the sonic process does not give homogeneous dispersions of larger vesicles and can degrade the complex lipids and other components of the liposomes. The single filtration step disclosed in FR 2298318A still provides relatively random size particles.

The preparation of liposomes and their use in drug therapy has been previously described. See, for instance, U.S. Patent 4,053,585; Geman Patent 2,532,317; Netherlands application 73/04133; and Biochemistry 16 (12) 2806 (1977).

According to the present invention, there is provided a method for the production of liposomes of uniform size comprising forming liposomes in relatively random sizes and decreasing their size by extruding the random sized liposomes through at least one orifice at a pressure of at least about 1170 bar.

The present invention thus provides processes by which liposomes of uniform size and composition, and with predictable properties may be produced. The initially random sized liposomes are subjected, preferably to extrusion through a plurality of successive orifices of decreasing size. This extrusion is preferably the final step of the process, to provide a product ready for use.

By a method embodying the invention the liposomes, in which the bis-anthracyclines may be encapsultated are produced as follows:

Those agents which are to compose the lipid membrane of the liposome, such as phospholipids, cholesterol and/or other biologically active or inactive amphiphiles, or macromolecules are mixed in an organic solvent such as ethers, chloroform, alcohol, etc. and then dried onto the interior surface of a vessel under a vacuum. As an example, phosphatidic acid L-alpha-lecithin and cholesterol were mixed into a solution of 7:3:1 chloroform:isopropanol:methanol respectively and vacuum dried. An aqueous solution of the drug was added to the dried lipids at a temperature above the phase transition temperature of the lipid mixture. In this example, a bis-anthracycline at 1 mg/ml in isotonic phosphate buffer was added and the solution rolled with the lipids for one hour to allow slow hydration.

The resulting liposome size was greater than 0.5 μm in diameter (light scattering method). These mechanically dispersed liposomes in which the drug is incorporated, are then passed through orifices provided by a Nucleopore type filter (uniform pore size) starting with 1.00 μm and going successively down to the desired vesicle size (e.g. 0.1 μm). If the lipid concentration of these liposomes was greater than 10 mgm/ml the process was repeated for maximum uniformity. Following these steps the untrapped drug was removed from the vesicles by dialysis and the drug-containing vesicles were collected for further use.

If the liposome size is desired to be less than 0.1 to 0.05 μm the liposomes are then subjected to an extrusion under a pressure at a pressure of at least about 1170 bar through a small orifice. For example, the liposomes were extruded using a French Press and Pressure Cell (Aminco type) maintained at about 1170 bar during the entire extrusion. The extrusion at this pressure may be repeated for enhanced uniformity of resulting vesicles and very uniform liposomes can be easily and reproducibly made by this process. The extrusion pressure, orifice size, and temperature can be used to control the size of the liposome. Extrusion may be at pressures up to 2070 bar.

Subsequent to the extrusion, the free untrapped drug can be removed readily by dialysis leaving a uniform, stable liposome population containing the drug.

As noted the liposome wall material may be any desired lipid, such as phospholipids, cholesterol, etc. Such liposomes may be produced, as noted, in closely controlled sizes; and, in addition depending on the lipid employed, with positive or negative charges thereon.

Utilizing controlled liposome size, material and charge, it has been determined that in the mammalian organism, the liposomes will preferably collect in particular organs, such as lung, liver, spleen, etc. Thus, the encapsulated drug may be delivered to specific sites within the organism. It will be apparent that

utilization of the liposomes for such purposes, facilitates the effectiveness of the drug in contacting tissues at selected sites since the drug will concentrate at the selected sites. At the same time, the drug concentration throughout the general body tissues will be greatly lowered to reduce undesirable side effects.

Enhanced effectiveness of Bis-anthracyclines in liposomes vs free bis-anthracyclines

The bis-anthracyclines mentioned are generally more effective against mouse cancer than the parent mono-anthracycline, such as Daunorubicin, and effective clinical mono-anthracycline. On the other hand, the bis-anthracyclines were less potent and less effective than would be expected when extrapolated from the comparative tests in which the bis-anthracycline was found to be over 100 times as potent as Daunorubicin. If this discrepancy is due to the lower transport of the very large bis-anthracycline molecule into cells or to the target cellular receptor, then overcoming this possible barrier should enhance the drug effectiveness of bis-anthracylines.

Daunorubicin and Type I and/or Type II bis-anthracyclines (see EP—B—4467) were encapsulated in liposomes of the small unilamellar class, composed of phosphatidic acid, L-alpha lecithins, and cholesterol. These uniform small unilamellar vesicles were prepared by a final step of extrusion from a French Press at a pressure of 1170 bar.

The liposome encapsulated Type II bis-anthracycline drugs and free drugs were compared for their capacity to kill leukemic cells. The free drugs were equally active and equally potent when Daunorubicin and the bis-anthracyclines were compared for the cell kill of L1210 leukemia cells. Daunorubicin was found to change only a few % (not significantly) between acting as a free drug or being liposome encapsulated (the I_{50} was about 0.20 micromolar in both cases. However, the encapsulated bis-anthracycline was very much improved in antileukemic activity when liposome encapsulated as noted in Table 1:

TABLE 1

Improved activity of liposome encapsulated bis-anthracyclines

State	I_{50} Leukemic cell kill
Free drug	0.250 micromolar
Liposome encapsulated	0.003 micromolar

In addition, Daunorubicin or bis-daunorubicin incorporated into these same liposomes and administered into BDF$_1$ mice carrying P—388 leukemia, could be given at more than 2 times the lethal dose of the free drug without producing a lethal effect. Under these conditions they were still effective anti-leukemic drugs in vivo, showing that such encapsulation produced a therapeutic advantage in making the doses less toxic. Whereas the therapeutically effective injected dose of the Type II bis-anthracyclines in treating murine leukemia may be 10—50 mg/kg on a q 4 d schedule, when the bis-anthracycline is incorporated into these same phosphatidic acid-lecithins-cholesterol liposomes, it is 2 to 8 mg/kg or 5 fold less. Thus it has been observed that the same liposome that lowers the risk of anthracycline toxicity can enhance the potency of the bis-anthracyclines. The combination of these effects, increasing the dose needed to produce toxicity and increasing potency or lowering the dose required to achieve a therapeutically useful effect in treating in vivo murine leukemia, is called enhancing the therapeutic index of a drug. We have thus seen that incorporation into liposomes enhances or improves the therapeutic index of both mono anthracyclines, such as those now used for treating human diseases, and the new bis-anthracyclines.

Directing drugs to specific or selective tissues in the mammalian species through incorporation in liposomes has been demonstrated. Table 2 below presents data in this regard and illustrates the effect of liposome size upon concentration in various tissues.

TABLE 2

% Dose[a] in Selected tissues at various times after IV administration of size I and size II[b] liposomes to mice

Time hours	Tissue					
	Liver		Spleen		Lung	
	Size I	Size II	Size I	Size II	Size I	Size II
after 1	19.8	24.1	4.5	5.0	$\boxed{8.5}$	1.0
after 5	8.3	$\boxed{20.5}$	2.9	2.7	$\boxed{5.7}$	0.5
after 24	0.5	0.7	0.3	0.3	$\boxed{5.2}$	0.1

[a]The drug used was cytosine arabinoside. The liposomes in both cases were composed of phosphatidyl choline, phosphatidyl serine, and cholesterol in the ratio 5:1:5.
[b]Size I was extruded to yield approximately 1.2 µm liposomes. Size II was extruded to yield approximately 0.5 µm liposomes.

Table 2 shows that size I liposomes accumulate in lung tissue; whereas no difference with respect to spleen tissue is noted. At early times, size II liposomes, on the other hand, preferentially accumulate in liver tissue.

Claims

1. A method for the production of liposomes of uniform size comprising forming liposomes in relatively random sizes and decreasing their size by extruding the random sized liposomes through at least one orifice at a pressure of at least about 1170 bar.
2. A method according to claim 1 wherein the or each extrusion is carried out in the presence of a therapeutic agent.
3. A method according to claim 2, which includes the additional step of separating liposomes having encapsulated therapeutic agent from unencapsulated therapeutic agent.
4. A method according to any one of the preceding claims, wherein the or a last said extrusion of succesive extrusions is the final step in the preparative process.
5. A method according to any one of the preceding claims, wherein the liposomes are forced through at least one said orifice at a pressure up to 2070 bar.
6. A method according to any one of the preceding claims, wherein the said liposomes are composed of lipids including phospholipids, macromolecules, cholesterols, amphiphiles, or a mixture thereof.
7. A liposome made by the method of any one of the preceding claims and incorporating a bis-anthracycline as claimed in European Patent No. 4467.

Patentansprüche

1. Verfahren zur Herstellung von Liposomen regelmäßiger Größe unter Bilden von Liposomen relativ unregelmäßiger Größe und Verkleinern ihrer Größe durch Extrudieren der unregelmäßig großen Liposome durch wenigstens eine Düsenöffnung bei einem Druck von mindestens etwa 1170 bar.
2. Verfahren nach Anspruch 1, worin die oder jede Extrusion in Gegenwart eines therapeutischen Mittels durchgeführt wird.
3. Verfahren nach Anspruch 2, das den zusätzlichen Schritt der Trennung von Liposomen mit eingekapseltem therapeutischem Mittel von nicht eingekapseltem therapeutischem Mittel einschließt.
4. Verfahren nach einem der vorhergenden Ansprüche, worin die oder eine letzte Extrudierung der aufeinanderfolgenden Extrudierungen der letzte Schritt im Herstellungsverfahren ist.
5. Verfahren nach einem der vorhergenden Ansprüche, worin die Liposome durch wenigstens eine Düsenöffnung bei einem Druck bis zu 2070 bar gepreßt werden.
6. Verfahren nach einem der vorhergehenden Ansprüche, worin die Liposome aus Lipiden, einschließlich Phospholipiden, Makromolekülen, Cholesterinen, Amphiphilen oder einem Gemisch hiervon zusammengesetzt werden.
7. Liposom, hergestellt nach dem Verfahren eines der vorhergehenden Ansprüche und ein Bis-anthracyclin, wie in dem Europäischen Patent Nr. 4467 beansprucht, enthaltend.

Revendications

1. Procédé de production de liposomes de taille uniforme, consistant à former des liposomes à des

tailles relativement statistiques et à diminuer leur taille par extrusion des liposomes de taille statistique à travers au moins un orifice à une pression d'au moins environ 1170 bars.

2. Procédé selon la revendication 1, où la ou chaque extrusion est effectuée en présence d'un agent thérapeutique.

3. Procédé selon la revendication 2, qui comprend l'étape supplémentaire de séparer les liposomes où est encapsulé l'agent thérapeutique, de l'agent thérapeutique non encapsulé.

4. Procédé selon l'une quelconque des revendications précédentes, où l'extrusion ou la dernière extrusion desdites extrusions successives est l'étape finale du procédé de préparation.

5. Procédé selon l'une quelconque des revendications précédentes, où les liposomes sont forcés à travers au moins un orifice à une pression pouvant atteindre 2070 bars.

6. Procédé selon l'une quelconque des revendications précédentes, où lesdits liposomes se composent de lipides comprenant des phospholipides, des macromolécules, des cholestérols, des amphiphiles, ou un mélange.

7. Liposome préparé par le procédé selon l'une quelconque des revendications précédentes, où est incorporée une bis-anthracycline telle que revendiquée dans le Brevet Européen N° 4467.